VIROLOGY 213, 413–424 (1995)

HIV-1 Tat Directlv Interacts with the Interferon-Induced. Double-Stranded

NIGEL A. J. MCMILLAN,* RENE F. CHUN,† DAVID P. SIDEROVSKI,‡ JULIEN GALABRU,§, W. MARK TOONE,* CHARLES E. SAMUEL,¶ TAK W. MAK,‡ ARA G. HOVANESSIAN,§ KUAN-TEH JEANG,† and BRYAN R. G. WILLIAMS*,[1]

*Department of Cancer Biology, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195; ‡Amgen Institute, Ontario Cancer Institute, and Department of Medical Biophysics, University of Toronto, 500 Sherbourne Street, Toronto, Ontario, Canada M4X 1K9; and §Unit of Virology and Cellular Immunology (UA CNRS 1157), Institut Pasteur, 25, Rue du Dr Roux, 75724 Paris Cedex 15, France, ¶Department of Biological Sciences, University of California, Santa Barbara, California 93106; and †Laboratory of Molecular Microbiology, NIH, 9000 Rockville Pike, Bethesda, Maryland 20892

Received May 11, 1995; accepted August 18, 1995

We present evidence that the HIV-1 Tat protein and the RNA-dependent cellular protein kinase, PKR, interact with each other both *in vitro* and *in vivo.* Using GST fusion chromatography, we demonstrate that PKR, interacts directly with the HIV-1 Tat protein. The region in Tat sufficient for binding PKR maps within amino acids 20 to 72. In *in vitro* assays, the two-exon form of Tat (Tat 86) was phosphorylated by PKR, while the one exon form of Tat (Tat 72) inhibited PKR autophosphorylation and substrate phosphorylation. The ability of Tat to interact with PKR was demonstrated in both yeast and mammalian cells. Expression of PKR in yeast results in a growth suppressor phenotype which was reversed by coexpression of a one exon form of Tat. Expression of Tat 72 in HeLa cells resulted in direct interaction with PKR as detected by coimmunprecipitation with a Tat antibody. Tat and PKR also form a coimmunoprecipitable complex in cell-free extracts prepared from productively infected T lymphocytes. The interaction of Tat with PKR provides a potential mechanism by which HIV could suppress the interferon system.

INTRODUCTION

One of the intriguing features of HIV infection of humans is the presence of increasing serum levels of the antiviral agent, interferon (IFN), as the disease progresses (Read *et al.,* 1985). Increased levels of the interferon-induced enzyme 2–5A synthetase, which plays a role in the antiviral effects of interferon (Rysiecki *et al.,* 1990), have also been correlated with disease progression (Read *et al.,* 1985). Thus HIV appears to have evolved mechanisms for overcoming inhibition by interferon. Paradoxically, treatment of many cell types, including peripheral blood lymphocytes, monocytes, macrophages, and T cells, with IFN has been shown to inhibit HIV type 1 (HIV-1) infection (Dolei *et al.,* 1986; Gendelman *et al.,* 1990; Hartshorn *et al.,* 1987; Ho *et al.,* 1985; Kornbluh *et al.,* 1989, 1990; Poli *et al.,* 1989; Yamada *et al.,* 1988; Yamamoto *et al.,* 1986), although the molecular mechanisms involved appear to differ depending upon cell type.

IFN treatment of mammalian cells results in the establishment of an antiviral state mediated at least in part by the interferon-induced, double-stranded RNA-activated protein kinase, PKR (for reviews see Hovanessian, 1989; Samuel, 1991). PKR is found in most cell types and is induced 5- to 10-fold upon exposure to interferon (Galabru and Hovanessian, 1985; Samuel, 1991). It is activated by low concentrations of double-stranded (ds) RNA, resulting in autophosphorylation of several serine residues on the kinase molecule, while high concentrations of dsRNA are known to inhibit PKR activation (Galabru and Hovanessian, 1987; Galabru *et al.,* 1989). Once activated, PKR is then able to phosphorylate its major substrate, the α subunit of eukaryotic initiation factor 2 (eIF2α) (Hovanessian, 1989). This results in rapid inhibition of protein synthesis due to the sequestering and inactivation of the guanine nucleotide exchange factor, eIF2B, by the phosphorylated eIF2α (Safer, 1983; Hershey, 1989).

The gene encoding PKR has been cloned and characterized (Meurs *et al.,* 1990). PKR contains all of the protein kinase subdomains within the C-terminal region, while the N-terminal region has been shown to be important for the binding of dsRNA (Barber *et al.,* 1991; Chong *et al.,* 1992; Feng *et al.,* 1992; Katze *et al.,* 1991; Patel and Sen, 1992). PKR shares homology at the C-terminal end with two other eukaryotic eIF2α kinases, the heme-controlled repressor and GCN2. Alignment of these sequences indicates a common insert region between kinase subdomains IV and V which may define a common eIF2α binding site (Chong *et al.,* 1992; Ramirez *et al.,* 1992). PKR has been expressed in *Saccharomyces cere-*

[1] To whom correspondence and reprint requests should be addressed at Department of Cancer Biology — NN10, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195. Fax: (216) 445-6269. E-mail: williab@cesmtp.ccf.org.

0042-6822/95 $12.00

visiae, resulting in a growth suppression phenotype due to the phosphorylation of yeast eIFα (Chong *et al.,* 1992; Dever *et al.,* 1992, 1993). This slow-growth phenotype can be reversed by the coexpression of either the N-terminal half of PKR or mutant yeast eIF2α (Chong *et al.,* 1992). In accord with this growth suppressor activity in yeast, expression of inactive kinase mutants in NIH3T3 cells resulted in cell transformation and tumor formation in a nude mouse assay (Koromilas *et al.,* 1992, Meurs *et al.,* 1993).

HIV transactivation-responsive RNA has been shown to activate PKR *in vitro* due to its extensive secondary structure (Edery *et al.,* 1989; Maitra *et al.,* 1994; Roy *et al.,* 1991; SenGupta and Silverman, 1989), although one report suggests an inhibitory activity (Gunnery *et al.,* 1990). Previous work has suggested that HIV infection reduces the cellular level of PKR (Roy *et al.,* 1990). This reduction was also observed in HeLa cells stably expressing normal HIV-1 Tat but not a Tat mutant. However, the mechanism by which continual expression of Tat decreases PKR levels was left unresolved. Tat stimulates efficient HIV gene expression by interacting with the transactivation-responsive (TAR) region found in all HIV mRNAs, and hence is essential for HIV replication (Arya *et al.,* 1985; Drysdale and Pavlakis, 1991; Huang *et al.,* 1994; Rosen, 1991). Two forms of Tat are synthesized during HIV-1 infection, the two-exon form (86 amino acids for LAI/Bru HIV 1 derivatives, approximately 101 amino acids for all others) made in the absence of HIV Rev activity (early expression) and a single exon, 72-amino-acid protein that appears in the presence of Rev activity (late expression) (Malim and Cullen, 1991). Both forms of Tat (Tat 72 and Tat 86–101) are fully functional in transactivating the HIV LTR (long terminal repeat) (Sodroski *et al.,* 1985; Tiley *et al.,* 1990; Weeks *et al.,* 1990). Tat also interacts with cellular proteins such as the Tat binding protein, TBP-1 (Nelbock *et al.,* 1990), MSS-1 (Shibuya *et al.,* 1992), and Sp1 (Jeang *et al.,* 1993a,b), although the functional implications of these interactions remain to be determined. Tat may also play functional roles in addition to transcription (Huang *et al.,* 1994).

As PKR has previously been shown to be activated by HIV TAR RNA *in vitro* (Edery *et al.,* 1989; Maitra *et al.,* 1994; Roy *et al.,* 1991; SenGupta and Silverman, 1989) and the expression of Tat protein correlated with a reduction in PKR activity (Roy *et al.,* 1990), we investigated the possibility that Tat was interacting directly with PKR. Here we show that Tat binds directly to PKR. Interestingly, while Tat 72 inhibited the activation of PKR, Tat 86 was phosphorylated by PKR. Preincubation of PKR with eIF2 blocked Tat protein binding and the inhibition of PKR activity by Tat 72. The interaction was also manifested in HeLa cells where functional Tat 72 bound cellular PKR, as indicated by coimmunoprecipitation. *In vivo* interaction could also be demonstrated in *Saccharomyces cerevisiae,* where the PKR-mediated repression of growth was reverted by coexpression of single-exon Tat, and in productively infected T lymphocytes.

MATERIALS AND METHODS

Plasmids and proteins. pGEX-PKR was created by inserting the 2-kb *Hin*dIII cDNA fragment (Meurs *et al.,* 1990) into the *Sma*I site of pGEX2T. pGEX-6M was similarly cloned, but using the Lys^{296}–Arg mutant previously described (Chong *et al.,* 1992). To create pLTR3T4hyg, the SFFV LTR of pSFSVn-LC7 (Ballhausen *et al.,* 1988) was removed by *Eco*RI–*Sac*I digestion and replaced by blunt-end ligation with a functional HIV-1 3′ LTR (nts 8443–9162 of HIV isolate HXB2); the *neo* gene of the resultant plasmid was removed by complete *Hin*dIII and partial *Bam*HI digestion and replaced by the 2.2-kb *Hin*dIII–*Bam*HI fragment of pGEMSV2hyg (Siderovski *et al.,* 1992), containing the *hyg* gene and SV40 polyadenylation signal. Plasmids pMAMTat86, pMAMTat72, and pMAMTat72AS contain the 286-bp *Xho*I–*Sal*I cDNA fragment of pGEMSV2Tat^{wt} (Siderovski *et al.,* 1992) and the 1.3-kb *Sal*I–*Pvu*II fragment of *Tat* exon 1 (nts 5342–6631 of HIV isolate HXB2) in sense and antisense orientation, respectively, each cloned within the *Sal*I site of pMAMneo (Clontech, Palo Alto, CA). The plasmid yex4Tat(1–67) was created by inserting the 240-bp *Sal*I–*Hin*dIII fragment from the HIV-1 genomic clone pNL43 in the *Sal*I–*Hin*dIII of yeast expression vector pEMBLyex4. This plasmid contains the *URA3* gene and the GAL10-CYC1 hybrid promotor and codes for the first 67 amino acids of the HIV *Tat* gene. Plasmid yex4Tat86 was created by inserting the 290-bp *Bgl*II–*Sal*I fragment of Tat cDNA M910 (Siderovski *et al.,* 1992) into the *Bam*HI–*Sal*I site of pEMBLyex4. Plasmid 86H has been described elsewhere (Chong *et al.,* 1992). It contains the *HIS3* gene and the human *PKR* gene under the control of the GAL10-CYC1 hybrid promotor. Tat 72 was synthesized by Dr. Clark-Lewis and corresponds to the amino acid residues 1 to 72 of the LAI/BRU isolate sequence. Recombinant Tat 86 (product of the first and the second exons of the *tat* gene) and recombinant Rev proteins were from American Bio-Technologies Inc. and were provided through the AIDS Research and Reference Reagent Program, Division Reagent Program, of AIDS, NIAID, NIH. SDS–PAGE analysis and visualization by silver staining indicated that Tat 86 and Tat 72 proteins were greater than 95% pure. Histones H2A and H3 were from Sigma.

Cell lines. HeLa S3 cells (ATCC CCL 2.2) were obtained from the American Type Culture Collection (Rockville, MD). All HeLa cell lines were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 μg/ml penicillin, 100 μg/ml streptomycin, and appropriate G418 and hygromycin-B concentrations (see below). The cell line HeLa LTRT4 was obtained by calcium-phosphate-mediated transfection of HeLa S3 with pLTRL3T4hyg and

subsequent hygromycin-B selection (300 g/ml). HeLa cell lines LTRT4/Tat86, LTRT4/Tat72, and LTRT4/Tat72AS were created by liposome-mediated transformation (Felgner *et al.,* 1987) of HeLa LTRT4 with pMAMTat86, pMAMTat72, and pMAMTat72AS, respectively, and subsequent selection in medium containing 700 μg/ml G418 (active) and 300 μg/ml hygromycin-B. MT-2 cells infected with HIV-1 (NL4–3) were cultured as described previously (Jeang *et al.,* 1993a).

In vitro autophosphorylation assay. PKR was purified from interferon-treated Daudi cells using a specific monoclonal antibody (MAb 71/10) with protein G Sepharose and assayed for protein kinase activity as previously described (Hovanessian *et al.,* 1987). Briefly, for the activation of PKR, immune complex preparations were incubated in buffer C (20 m*M* HEPES, pH 7.5, 50 m*M* potassium chloride, 5 m*M* 2-mercaptoethanol, 1.5 m*M* magnesium acetate, and 1.5 m*M* manganese chloride) containing poly(I):poly(C) (1 μg/ml) and 2 μM [γ-^{32}P]ATP (50 Ci/mmol) at 30°C for 15 min. For the phosphorylation of exogenous substrates, after PKR phosphorylation the samples were washed before incubation (15 min, 30°C) in buffer C containing [γ-^{32}P]ATP (as above) and the exogenous substrate (eIF2, Tat 86, or histone H2A). Eukaryotic initiation factor 2 purified to 85% homogeneity was a gift from Dr. William Merrick. Reactions were stopped by the addition of twofold concentrated electrophoresis buffer and the samples were analyzed by 10 or 15% SDS–PAGE. ^{32}P-labeled PKR, H2A, and Tat 86 were visualized by autoradiography.

Expression of glutathione-S-transferase–PKR fusion protein. The plasmid pGST–PKR or pGST–6M were transformed into the *Escherichia coli* strain DH5α. Overnight cultures (50 ml) of uninduced cells were harvested by centrifugation at 5000 *g* and resuspended in ice-cold PBS. Cells were lysed by sonication (3 pulses of 15 sec at 20,000 cycles) and centrifuged at 12,000 *g* before the addition of 100 μl glutathione–Sepharose beads (Pharmacia). Cell extracts were incubated at 4°C for 15 min and the beads washed five times with ice-cold PBS and stored at −70°C. GST–Tat or GST–Tax fusion proteins were similarly prepared.

Protein binding assays between PKR and HIV-1 Tat. For these assays glutathione beads were pretreated with 0.5% skim milk in buffer C prior to PKR purification. Binding assays were performed by washing the GST–PKR/glutathione–Sepharose beads twice in buffer C followed by the addition of 1 μg of Tat 72 protein. Incubation was continued on ice for 30 min before extensive washing with buffer C supplemented with 0.5% NP 40. The beads were treated with SDS–PAGE buffer and boiled for 3 min and the eluted proteins separated by SDS–PAGE. GST (Pharmacia), GST–Tat (amino acids 1 to 101) fusion or GST–Tax fusion proteins were bound to glutathione–Sepharose 4B beads. The protein–beads were reacted separately with cell extracts and then washed extensively (more than 20 column volumes) with buffer (20 m*M* HEPES, pH 7.9 [KOH], 1 m*M* $MgCl_2$, 17% glycerol, 2 m*M* DTT) containing 0.1 M KCl (wash). Subsequent to washing, the sepharose beads were eluted stepwise with buffer containing 0.25 *M* KCl and then 0.5 *M* KCl. Eluates were desalted and concentrated using a 10,000 MW cutoff microconcentration tube (Amicon) resolved by SDS–PAGE.

Western blotting. Proteins were separated by SDS–PAGE and blotted to polyvinyl difloride membrane (Immobilon-P, Millipore Corp.) according to the manufacturers instructions. Blots were reacted with Tat polyclonal antibody (705, provided by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Bryan Cullen) or PKR polyclonal or monoclonal antibody and detection was performed using a peroxidase-labeled secondary antibody and enhanced chemiluminescence (ECL) (Amersham).

Expression of HIV-1 Tat in Saccharomyces cerevisiae. Plasmids were transformed into a yeast strain W303a (MATa, *can*1-100, *his*3-11,15, *leu*2-3, 112, *trp*1-1, *ura*3-1, *ade*2-1) already containing PKR (86H) under the GAL10-CYC-1 promoter on the centromeric CEN/ARS vector pRS313, as previously described (Chong *et al.,* 1992). Expression of PKR and Tat was induced by growth on 2% galactose plates. All plasmids were maintained by selection based on the prototrophic markers contained within each plasmid.

Yeast protein preparation. Yeast strains were grown in minimal medium (0.67% Bacto yeast nitrogen base without amino acids, 0.1% glucose, and tryptophan and leucine added to 20 μg/ml) at 30°C to an OD_{600} of 1.0. Following extensive washing the cells were resuspended in minimal medium containing tryptophan and leucine at 20 μg/ml with 2% galactose as the carbon source and the incubation was continued at 30°C. Cells were harvested after 24 hr, washed in ice-cold water, and resuspended in 1 ml of disruption buffer (20 m*M* Tris–HCl, pH 8, 10 m*M* $MgCl_2$, 1 m*M* EDTA, 5% glycerol, 1 m*M* DTT, 0.3 *M* ammonium acetate, and 1 m*M* PMSF). The cells were disrupted by vortexing five times for 1 min in the presence of 1/2 volume of glass beads and the supernatant was decanted and centrifuged for 60 min at 12,000 *g*.

Immunofluorescence analysis. Adherent HeLa cell cultures were collected in calcium- and magnesium-free phosphate-buffered saline (PBS) containing 0.6 m*M* EDTA, stained with phycoerythrin-conjugated anti-L3T4 monoclonal antibody (Becton–Dickinson, San Jose, CA) for 1 hr at 4°C in 100 μl PBS + 2.5% FCS, washed with cold PBS, and analyzed by single-color flow cytometry on a FACScan (Becton–Dickinson).

Preparation of HeLa cell extracts. Cell extracts from confluent 100-mm plates were prepared as previously described (Hovanessian *et al.,* 1987) except that Buffer I (20 m*M* Tris–HCl, pH 7.6, 50 m*M* KCl, 400 m*M* NaCl, 1

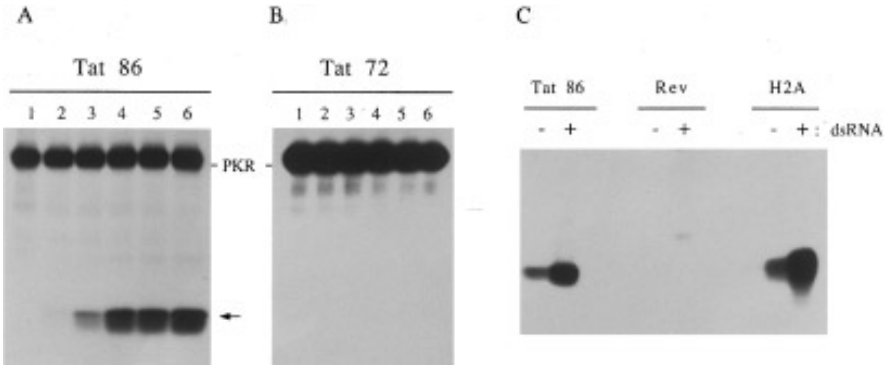

FIG. 1. HIV-1 Tat 86 is phosphorylated specifically by activated PKR. Monoclonal antibody-bound PKR was first activated (1 μg per sample) before the addition of Tat 86 (A) or Tat 72 (B) at different concentrations : 0, 0.05, 0.1, 0.2, 0.4, and 1 μg in lanes 1–6, respectively. The arrow indicates the position of Tat 86. In (C) PKR preparations were first activated in the absence or presence (−/+ lanes) of poly(I):poly(C) before the addition of different substrates : Tat 86 (1 μg), Rev (1 μg), and histone H2A (5 μg). Only the section of the gel corresponding to the positions of Tat 86 (14 kDa), Rev (18 kDa), and histone H2A (15 kDa) is presented. Note: B was overexposed to show the lack of phosphorylation of Tat 72.

m*M* EDTA, 1% Triton X-100, 20% glycerol, and 5 m*M* 2-mercaptoethanol) contained 40 m*M* NaCl and 10 μg/ml leupeptin. Equal amounts of total protein were reacted with a monoclonal antibody to PKR (71/10) in the low salt Buffer I for 16 hr before the addition of protein G–Sepharose. Incubation was continued for 60 min before the Sepharose beads were washed three times in Buffer II (20 m*M* Tris–HCl, pH 7.6, 100 m*M* KCl, 0.1 m*M* EDTA, and 20% glycerol) and twice in Buffer III (Buffer II plus 2 m*M* $MnCl_2$ and 2 m*M* $MgCl_2$). The amount of PKR extracted was determined by Western blotting and equal amounts of kinase were used in kinase assays.

Coimmunoprecitation of Tat and PKR. HeLa cell extracts were prepared as described above using low salt Buffer I (Buffer I with 40 m*M* NaCl). Antibodies were added to 1 mg of cell extracts at a 1/1500 dilution and incubated at 4°C for 3 hr before the addition of protein A–Sepharose (Pharmacia) and the incubation was continued overnight. The immunocomplexes were washed three times in low salt Buffer I, resuspended in SDS–PAGE buffer, subjected to SDS–PAGE, and Western blotted as described above. PKR was detected using a polyclonal antibody to PKR provided by Drs. Michael Katze and Glenn Barber. Coimmunoprecipitation of Tat and PKR from cell-free extracts of MT-2 cells infected with HIV-1 (NL4-3) was performed on cells harvested at a time when more than 70% showed light microscopy-visible cytopathic effects from infection. The immunocomplexes were analyzed as described (Jeang *et al.,* 1993a).

RESULTS

HIV-1 Tat 86, but not Tat 72, is phosphorylated by PKR in vitro. PKR is a serine/threonine kinase that manifests two distinct protein kinase activities (Hovanessian, 1989), one for PKR activation through autophosphorylation and the other for phosphorylation of exogenous substrates which are generally basic proteins (Galabru and Hovanessian, 1987). Activation requires the binding of dsRNA to PKR molecules or, alternatively, single-stranded RNA molecules presenting internal dsRNA structures such as HIV TAR RNA (Galabru *et al.,* 1989; Roy *et al.,* 1991). Once PKR becomes autophosphorylated it is no longer dependent on its activator and can catalyze phosphorylation of exogenous substrates such as the α subunit of eIF2, histone H2A, or IκB (Galabru and Hovanessian, 1987; Kumar *et al.,* 1994).

Since Tat is a basic protein and has potential PKR phosphorylation sites within its sequence, we analyzed the capacity of activated PKR (i.e., autophosphorylated) to catalyze phosphorylation of one- and two-exon forms of Tat. Only the two-exon form (Tat 86) was found to be phosphorylated by PKR (Figs. 1A and 1B). The extent of phosphorylation of both Tat 86 and histone H2A was highly dependent on the prior activation of PKR by dsRNA, confirming that Tat 86 phosphorylation was mediated through PKR (Fig. 1C). Indeed, we have previously demonstrated that the degree of phosphorylation of histone H2A is directly correlated to the degree of activation of PKR (Galabru and Hovanessian, 1987). As a further control, the *rev* gene product Rev, a basic protein that appears to be phosphorylated during HIV infection (Rosen, 1991), was used in phosphorylation assays. However, Rev was not able to be efficiently phosphorylated by activated PKR, indicating specificity for the phosphorylation of Tat 86 by PKR.

The observation that Tat 86 was specifically phosphorylated by PKR suggested the presence of a Tat recognition site within PKR. Therefore the effect of Tat 72 on the two kinase functions of PKR: (1) autophosphorylation and (2) phosphorylation of the substrates Tat 86 and histone H2A, was investigated (Fig. 2). PKR samples (1 μg per assay) were mixed with different amounts of Tat 72 before being activated with dsRNA, and the level of kinase autophosphorylation was determined. Almost complete inhibition of autophosphorylation was observed at 0.2 μg

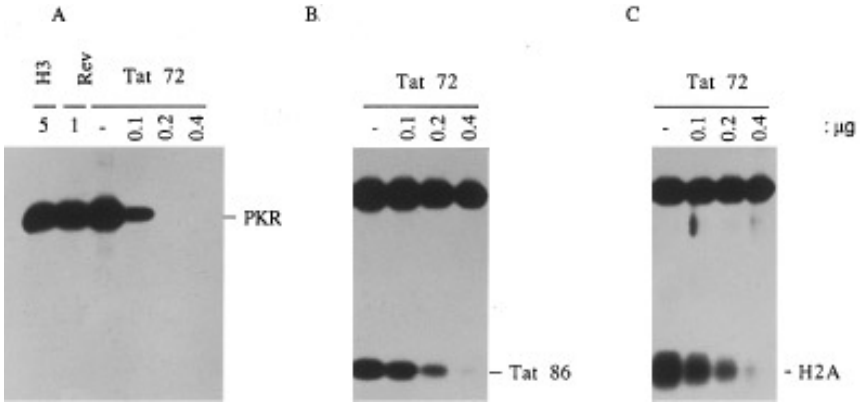

FIG. 2. Tat 72 inhibits the functioning of PKR. (A) Inhibition of PKR autophosphorylation by Tat 72. Monoclonal antibody-bound PKR (1 μg) was first incubated (5 min, 20°C) in the absence (−) or presence of histone H3 (5 μg), Rev (1 μg), or Tat 72 (0.1, 0.2, or 0.4 μg) before assay of PKR phosphorylation (see Materials and Methods). (B) Inhibition of PKR-catalyzed Tat 86 phosphorylation by Tat 72. Activated PKR preparations were washed in buffer C and incubated (5 min, 20°C) in the absence or presence of Tat 72 (0.1, 0.2, or 0.4 μg as indicated). The different samples were then assayed for the phosphorylation of Tat 86 (0.2 μg). (C) Inhibition of PKR-catalyzed histone H2A phosphorylation by Tat 72. Experimental procedure was as in B but histone H2A was used instead of Tat 86.

of Tat 72, a concentration that represents equal molar ratios of PKR and Tat. The observed inhibition did not appear to be a general property of basic proteins since neither Rev nor histone H3 caused inhibition of PKR autophosphorylation (Fig. 2A). To test whether Tat 72 could inhibit kinase substrate phosphorylation, PKR was preactivated by dsRNA prior to being mixed with Tat 72 and phosphorylation of the exogenous substrates Tat 86 (0.2 μg) or histone H2A (5 μg) was monitored. The addition of 0.2 μg of Tat 72 resulted in a dramatic reduction in substrate phosphorylation (Figs. 2B and 2C), thus indicating that Tat 72 is likely interacting with the same substrate recognition site as Tat 86 within PKR.

HIV-1 Tat binds directly to PKR. To further investigate the interaction between Tat and PKR, protein binding assays were performed. Monoclonal antibody-bound PKR, attached to protein G–Sepharose beads, was incubated with different amounts of Tat 72 before being washed extensively (in order to eliminate unbound protein). Equal aliquots of each sample were then assayed by immunoblotting, to reveal the presence of equal amounts of PKR protein, and by autophosphorylation to show the activation of PKR (Fig. 3). The phosphorylation of PKR was reduced substantially in relation to the amount of Tat 72 added during the preincubation period. As different samples were washed extensively after preincubation, the inhibition of PKR autophosphorylation was most likely due to binding of Tat 72.

The direct interaction of Tat with PKR was confirmed by determining the fragments of Tat sufficient for mediating interactions with PKR. This was achieved by analyzing for protein–protein complex formation using GST–Tat fusion-polypeptide chromatography. The binding of PKR to the GST–Tat column (Tat amino acids 1-101) was compared to the binding of PKR to similar columns made from either GST alone or GST–Tax fusion (Figs. 4A and 4B). (Tax, which has no sequence relatedness to Tat, is the 40-kDa trans-activator protein from HTLV-I.) Cell extracts were equilibrated with each affinity column matrix; bound proteins were then eluted with increasing concentrations of KCl and analyzed using either a monoclonal (Fig. 4B) or a polyclonal (Fig. 4A) anti-PKR serum. Both sera detected PKR in the GST–Tat 0.25 *M* KCl eluate (Fig. 4A, lane 8; Fig. 4B, lane 10), while no PKR was found in the GST alone or in GST–Tax samples (Figs. 4A and 4B).

To define further the region within Tat sufficient for interaction with PKR, deleted versions of the first coding exon of Tat were expressed as GST fusions (Fig. 4). In our experience, the laboratory strain of HIV-1 (i.e., HSB2 or NL4-3) that expresses the 1–86 amino acids of Tat (Myers *et al.,* 1994) and a molecularly engineered version of HIV-1 (NL4-3) that expresses exclusively the 1- to 72-amino-acid first coding exon of Tat (Chang, Neuvet, and

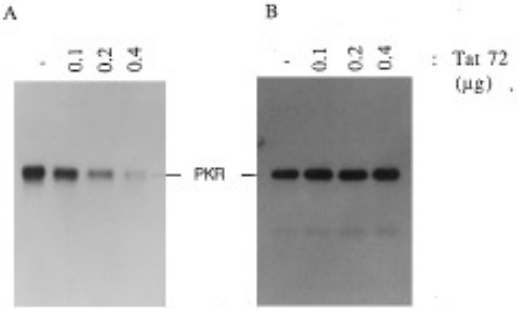

FIG. 3. Tat 72 binds to PKR and inhibits its activation. Monoclonal antibody-bound PKR was first incubated (10 min, 20°C) in buffer C containing different amounts of Tat 72 (as indicated). These preparations were then washed extensively in buffer C and separated into two equal aliquots for the assay of PKR phosphorylation (A) and absolute levels of PKR protein following immunoblotting using monoclonal antibody 71/10 and ^{125}I-labeled goat anti-mouse immunoglobulins (B).

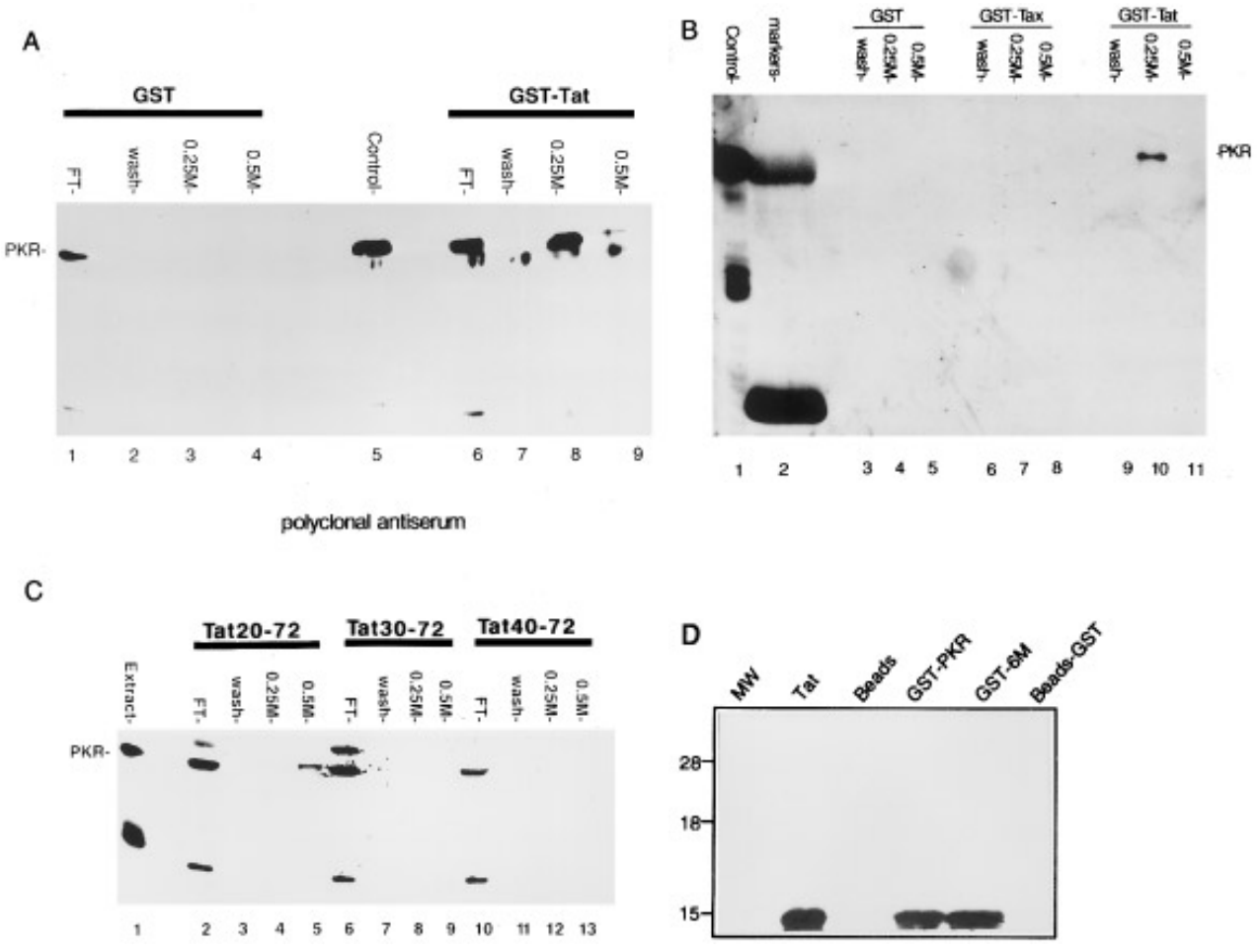

FIG. 4. (A,B,C) Retention of PKR in a GST–Tat protein column. GST (Pharmacia), GST–Tat (amino acids 1 to 101) fusion, or GST–Tax fusion proteins were bound to glutathione–Sepharose 4B beads. The protein beads were reacted separately with cell extracts, washed extensively with buffer (see Materials and Methods), and eluted stepwise with buffer containing 0.25 *M* KCl and then 0.5 *M* KCl. Eluates were desalted, concentrated, resolved by SDS–PAGE, transferred to PVDF-membrane, and probed with PKR-specific antisera. (A) Western blot analysis of column eluates using a polyclonal rabbit anti-PKR serum. FT lanes contain column flow-through material. (Lane 5) A aliquot of intact cell extract prior to reaction with beads. (Lanes 1–4) Elutions from GST–beads. (Lanes 6–9) Elutions from GST–Tat beads. PKR-, the position of a 68-kDa band. (B) Western blot analysis using a monoclonal anti-PKR serum. Elutions were performed with increasing salt (KCl) as indicated. Control lane contains whole extract. Markers indicate radiolabeled molecular weight standards. (Lanes 3–5) GST–beads; (lanes 6–8) GST–Tax beads; (lanes 9–11) GST–Tat beads. (C) Mapping of a subregion in Tat that binds PKR. Truncated versions of Tat protein (amino acids 20 to 72; amino acids 30–72, lanes 6–9; amino acids 40–72, lanes 10–13) were expressed as fusion proteins with GST and affixed onto glutathione–Sepharose 4B beads. These beads were reacted with cell extract (lane 1) followed by sequential washing and elutions. Samples were then analyzed by immunoblotting and detected using rabbit polyclonal anti-PKR serum. FT, "flow-through" material from each of the beads. Prominent PKR signal is seen in all three of the flow-through lanes (lanes 2, 6, and 10). A 68-kDa PKR band is detected in the 0.5 *M* eluate from the Tat 20–72 column. A higher exposure of the filter reveals a 10-fold lighter band in the same elution from Tat 30–72 but no signal in the Tat 40–72 elution. The smaller 48 kDa PKR-reactive band (lanes 1, 2, 6, and 10) is a presumed breakdown product of the 68-kDa polypeptide. (D) GST–PKR and GST–6M fusion proteins, bound to Sepharose beads (see Materials and Methods), were incubated with Tat 72 protein (1 μg) on ice for 30 min prior to extensive washing. Proteins were eluted by boiling, separated by SDS–PAGE, and Tat detected by Western blotting.

Jeang, in preparation) replicate well, although the 1–72 virus is slightly slower than the 1–86 virus in T lymphocytes. Hence, we reasoned that residues 72 to 101 would likely be dispensible for functional interactions with PKR. Indeed, we found that the PKR-interactive region in Tat was contained within the 20–72 (Fig. 4C, lane 5) stretch. Longer exposures revealed that Tat (30–72)–Sepharose retained, in its 0.5 *M* eluate, approximately one-tenth the amount of PKR seen for Tat (20–72)–Sepharose; while no PKR was seen in Tat (40–72)–Sepharose samples (data not shown). We noted on repeated comparisons that PKR was reproducibly retained by Tat (20–72)–Sepharose more avidly (0.5 *M* KCl eluate; Fig. 4C, lane 5) than by Tat (1–101)–Sepharose (0.25 *M* KCl eluate; Fig. 4A, lane 8; Fig. 4B, lane 10). One interpretation of this result is that the presentation of amino acids 20–72 in the context of GST–Tat 1–101 differs slightly from that in the setting of GST–Tat 20–72.

The direct binding of Tat 72 to PKR was also demonstrated in reciprocal experiments in which the presence

of Tat 72 protein was assayed by Western blot after incubation with purified PKR fused to glutathione-S-transferase (GST–PKR). Two forms of kinase fusion protein were prepared, the full-length wild-type kinase (GST–PKR), and a mutant kinase (GST–6M) which had lysine^{296} mutated to arginine (see under Materials and Methods). GST–PKR is a fully functional kinase able to activate and phosphorylate eIF2α (McMillan and Williams "unpublished data"), whereas GST–6M is unable to become activated as the mutated lysine is essential for phosphate transfer reactions (Chong *et al.,* 1992; Hanks *et al.,* 1988). The bacterially produced fusion proteins were immobilized on glutathione–Sepharose beads and purified prior to the addition of Tat 72. Following incubation with Tat 72, the beads were washed and the proteins eluted off the Sepharose beads and Tat 72 was detected by Western blotting. Tat 72 bound equally well to both GST–PKR and GST–6M (Fig. 4D) but was unable to bind to beads alone or beads with GST attached, thus indicating that PKR was mediating the binding of Tat protein.

PKR-mediated growth suppression in yeast is reversed by HIV-1 Tat. We have previously reported the development of a yeast system in which human PKR is expressed by a GAL10-CYC1 hybrid promoter on the vector pRS313 (Chong *et al.,* 1992). Strains of yeast transformed with this plasmid and grown on galactose as a sole carbon source exhibit a slow-growth phenotype due to the expression and activation of PKR. This results in phosphorylation of yeast eIF2α (Dever *et al.,* 1993). This phenotype can be rescued by coexpressing PKR with a mutant form of the yeast eIF2α (Ser^{51} → Ala), which can no longer be phosphorylated by PKR. Rescue of the slow-growth phenotype is also possible by coexpressing PKR with the amino terminus (amino acids 1–242) of PKR itself, a region known to bind dsRNA (Chong *et al.,* 1992). Accordingly, we have used this yeast system to investigate the role of Tat as an inhibitor of PKR. The advantage of using the yeast expression system is that, unlike mammalian cells, no endogenous PKR is present. *Saccharomyces cerevisiae* is known to contain an eIF2α kinase, GCN2, but its biology is markedly different from that of PKR in that it is thought to be activated by uncharged tRNAs in response to amino acid starvation (Dever *et al.,* 1992; Hinnebusch, 1988) although the exact activating species is unknown. Consequently, we used this heterologous kinase expression system to study an *in vivo* interaction between HIV-1 Tat and PKR.

HIV-1 Tat cDNAs (encoding amino acids 1–67 and 1–86) were cloned under the control of the GAL10-CYC1 promoter in the vector pEMBLyex4 and transformed into either the haploid yeast strain W303a or W303a already expressing human PKR under GAL10-CYC1 control (strain 86H). Expression of either Tat clone alone had no effect on growth (McMillan and Williams, unpublished data). Expression of PKR resulted in the previously observed slow-growth phenotype. However, when one exon

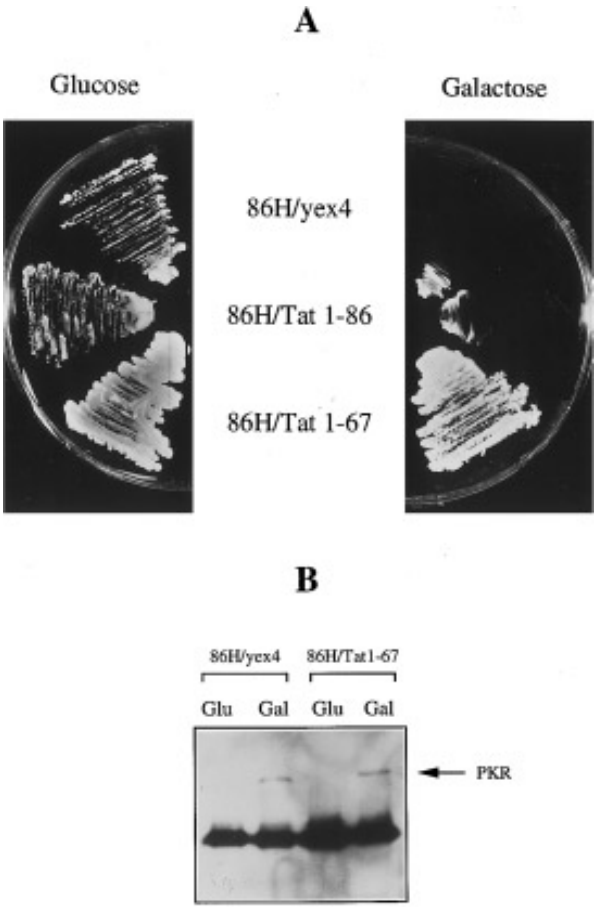

FIG. 5. Effect of Tat on PKR function in yeast. (A) Strains bearing plasmid 86H were cotransformed with plasmids encoding either Tat 1–67, Tat 1–86, or vector alone, and expression was induced by incubation on plates containing galactose as the sole carbon source. All plasmids were maintained by selection on synthetic medium lacking uracil and histidine. (B) PKR was immunopurified using monoclonal antibody 71/10 from yeast whole cell extracts prepared from the strains 86H/yex and 86H/Tat 1–67 (see Materials and Methods). Following extensive washing PKR was detected by Western blot. The arrows indicate the position of PKR. The other protein detected was IgG heavy chain from the monoclonal used to purify PKR.

Tat was coexpressed with the kinase, reversion of the slow-growth phenotype was observed (Fig. 5). Coexpression of Tat 86 had no effect on the slow-growth phenotype, in accord with its inability to inhibit PKR *in vitro* (Fig. 5). This inhibition of PKR function in yeast was not due to loss of PKR expression, as Western blots for PKR indicated similar levels of PKR in both 86H/yex- and 86H/Tat67-expressing strains (Fig. 5B). The expression of Tat72 in yeast gave the same phenotype as Tat67, i.e., reversion of slow growth.

HIV Tat interacts with endogenous PKR in a HeLa cell expression system. We wished to show a functional interaction between PKR and Tat protein in an *in vivo* context. In order to demonstrate a functional interaction in mammalian cells, a rapidly inducible Tat expression system was developed. HeLa cells were first stably transfected

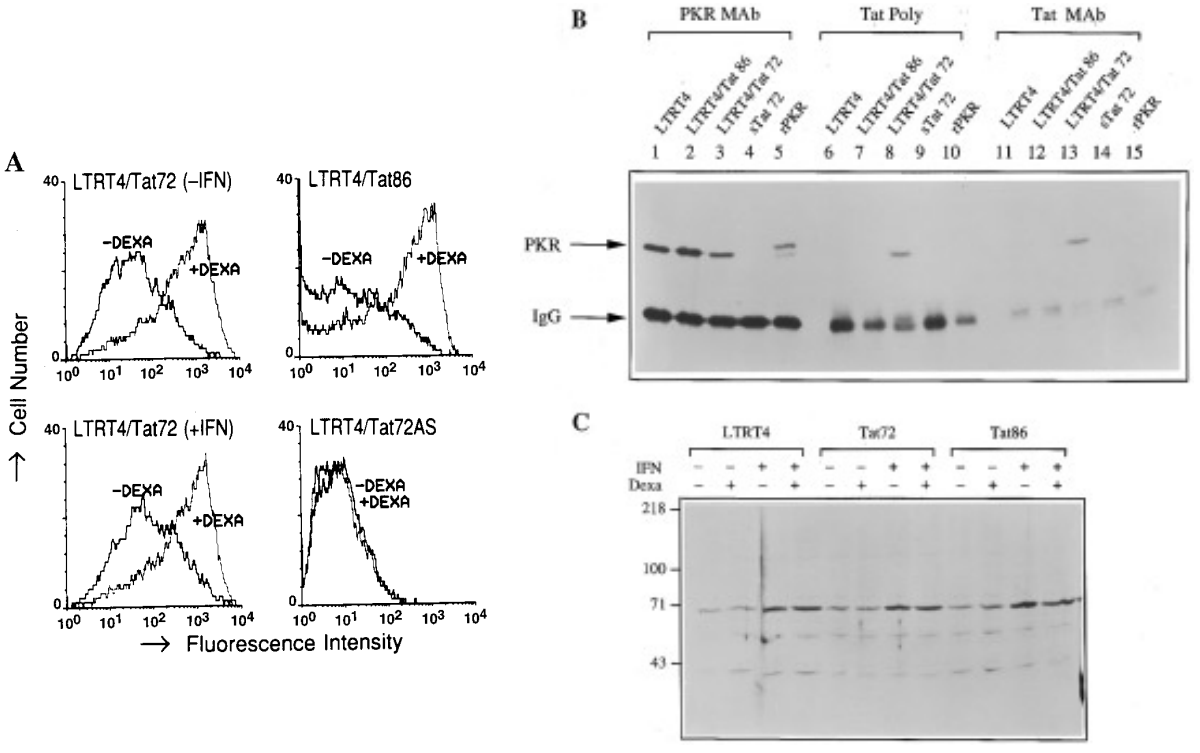

FIG. 6. (A) Interaction of PKR with Tat 72 *in vivo.* Immunofluorescence analysis of dexamethasone-induced L3T4 expression of HeLa cells conditionally expressing Tat. HeLa LTRT4/Tat72, LTRT4/Tat86, and LTRT4/Tat72AS cell lines were cultured for 18 hr in the presence (+DEXA) or absence (−DEXA) of 2 μM dexamethasone. HeLa LTRT4/Tat72 cells were treated (+IFN) or not treated (−IFN) with 500 U/ml human interferonα2 for 6 hr prior to dexamethasone addition. Cells were stained with a phycoerythrin-conjugated anti-L3T4 monoclonal antibody and cell surface fluorescence of 10^4 cells was measured by flow cytometry. (B) Cell extracts from interferon- and dexamethasone-treated HeLa cell lines (LTRT4, LTRT4/Tat72, and LTRT4/Tat86) were prepared under low salt conditions. Equal amounts of the extracts were then incubated with either PKR monoclonal (lanes 1–5), Tat polyclonal (lanes 6–10), or Tat monoclonal (lanes 11–15) antibodies. Synthetic Tat 72 and bacterially expressed mutant PKR (lys296 → arg) were used as controls. Following washing, immunocomplexes were analyzed for the presence of PKR by Western blot using a rabbit polyclonal PKR antibody. (C) The levels of PKR protein in HeLa cells is unaffected by the dexamethosone-induced expression of Tat. Equal amounts of whole cell extract (50 μg) were separated by SDS–PAGE, Western blotted, and PKR was detected using the monoclonal antibody 71/10.

with a Tat-dependent, cell-surface reporter construct, pLTRL3T4hyg, which expresses the murine T-cell antigen, L3T4, from a functional HIV-1 LTR containing the Tat-responsive region TAR. This cell line (HeLa LTRT4) was subsequently transformed with the plasmids pMAM-Tat86, pMAMTat72, or pMAMTat72AS, which express Tat 86, Tat 72, or an antisense *tat* mRNA, respectively, from the dexamethasone-inducible mouse mammary tumor virus LTR (Lee *et al.,* 1981). Of the resultant cell lines, HeLa LTRT4/Tat86 and LTRT4/Tat72 cells produced high levels of surface-bound L3T4 after 18 hr of dexamethasone treatment (Fig. 6A). This system reports on the expression of functional Tat, as upregulation of L3T4 expression was Tat-specific. Dexamethasone induction of HeLa LTRT4/Tat72AS cells did not induce expression of surface-bound L3T4 (Fig. 6A), indicating that expression was dependent on the synthesis of Tat protein. Prior interferon treatment was not observed to affect the dexamethasone induction of Tat expression in either HeLa LTRT4/Tat72 or LTRT4/Tat86 cells (Fig. 6A).

To test the interaction between Tat and PKR in these cell lines, PKR levels were induced by 6 hr pretreatment with 500 U/ml human interferon α2 and Tat expression subsequently induced by the addition of 2 μM dexamethasone. Protein extracts from interferon- and dexamethasone-treated cells were treated with antibodies to either PKR or Tat protein. Following extensive washing the immunocomplexes were Western blotted and analyzed for the presence of PKR (Fig. 6B). Synthetic Tat 72 and recombinant, bacterially expressed PKR were used as controls. PKR protein was present in all cell lines, as indicated by immunoprecipitation with PKR monoclonal antibody (Fig. 6B, lanes 1–3 and lane 5). PKR was not immunoprecipitated from either control cells or Tat 86-expressing cells by the anti-Tat antibodies. However, both the polyclonal and monoclonal Tat antibodies were able to coprecipitate PKR from the Tat 72-expressing cell line (Fig. 6B, lanes 8 and 13). This immunoprecipitation appears to be mediated by Tat 72, as recombinant PKR alone was not complexed by either of the Tat antibodies (Fig. 6B, lanes 10 and 15). Longer exposures of the Western blot indicated that a very low amount of PKR could

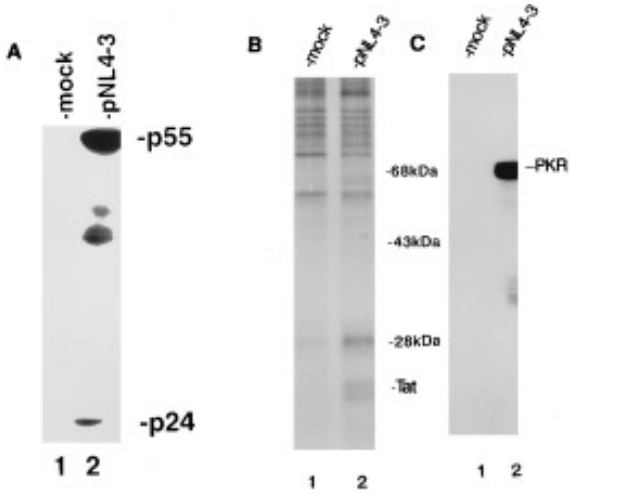

FIG. 7. Coimmunoprecipitation of Tat and PKR from cell-free extracts of infected T lymphocytes. MT-2 cells infected with HIV-1 (NL4–3) were harvested at a time when more than 70% showed light microscopy-visible cytopathic effects from infection. (A) Western blot analysis of total cell lysate using HIV-1 hyperimmune patient serum. (Lane 1) Mock-infected MT-2 cells; (lane 2) MT-2 infected with HIV-1 (NL4–3). p55 and p24 indicate Gag gene products. (B) A parallel set of infected cells was radiolabeled with [^{35}S]methionine and [^{35}S]cysteine and immunoprecipitated using a polyclonal rat anti-Tat serum (Yamamoto *et al.,* 1988). The position of a doublet that migrates with an approximate size of 14 kDa is indicated (−Tat). The lower band is presumed to be a degradation product of Tat. (C) Western blot analysis of anti-Tat immunoprecipitate from mock-infected (lane 1) and infected (lane 2) (but not radiolabeled) cells. Immunoprecipitates were resolved in a 10% SDS–polyacrylamide gel, transferred to PVDF-membrane (Millipore), and reacted with polyclonal rabbit anti-PKR serum (Huang *et al.,* 1994).

be immunoprecipitated from the Tat 86-expressing cells. This is consistent with Tat 86 being a substrate of PKR and being readily released by PKR, as has been previously observed for eIF2α (Hovanessian, 1989).

Finally, to rule out the possibility that the levels of PKR were affected by the various treatments, equal amounts of whole cell extract (50 μg) were immunoblotted and PKR was detected using the PKR monoclonal antibody 71/10 (Fig. 6C). As expected, PKR levels were observed to increase upon treatment of cells with interferon. However, neither the expression of Tat nor the dexamethasone treatment of cells was observed to affect the levels of PKR.

Tat associates with PKR in HIV-1-infected lymphocytes. To determine directly whether Tat and PKR interact within T lymphocytes, we analyzed cell-free extracts from HIV-1 (pNL4-3)-infected MT-2 cells for a coimmunoprecipitable Tat–PKR complex. Infected cells were divided into three parallel sets. One was analyzed in order to document viral-specific antigens as a measurement of infection (Fig. 7A). A second set was radiolabeled with [^{35}S]-methionine and [^{35}S]cysteine and immunoprecipitated with anti-Tat serum to verify recovery of Tat protein (Fig. 7B). A third was immunoprecipitated first with anti-Tat and then the immunoprecipitate was visualized by immunoblotting analysis using a PKR-specific antiserum (Fig. 7C). In the last set, a 68-kDa protein consistent with the molecular size of PKR was detected readily with the anti-PKR serum (Fig. 7C, lane 2).

DISCUSSION

Our results provide direct evidence that an HIV-encoded protein can directly bind to an IFN-induced antiviral protein and suggest a possible mechanism by which HIV-1 may interact with IFN system. Other functional differences have recently been described for the two forms of Tat. Tat 86 has been reported to specifically downregulate expression from the MHC class I gene promotor (Howcroft *et al.,* 1993) as well as be required for TAR-independent transactivation of the HIV envelope gene (Kim and Panganiban, 1993). The second coding exon of Tat also plays a role in the transactivation of integrated LTRs (Jeang *et al.,* 1993b). However, while single exon Tat (72 amino acids) is fully functional for transactivation of nonintegrated LTRs (Rosen, 1991), no specific function has been described to it that is distinct from that of two-exon Tat. The work presented here indicates that Tat 72 can act as a specific antagonist to both PKR activation and substrate phosphorylation. The latter activity occurred in manner independent of the inhibition of activation. This antagonistic activity can be shown to occur *in vitro* and in yeast, although it remains to be demonstrated during HIV infection.

Stable complexes between Tat 72 and PKR could be detected both *in vitro* and *in vivo,* indicating that inhibition of PKR function appears to be mediated via a direct protein–protein interaction. Thus the inhibition of PKR autophosphorylation by Tat 72 could be due to the hindrance of conformational changes that may be required for PKR activation. The region of Tat required for interaction with PKR was mapped to amino acids 20–72 (Fig. 4). Because Tat 40–72 contains the entire RNA-binding domain of Tat but this moiety did not bind PKR, our results are compatible with direct Tat–PKR association as opposed to an indirect interaction tethered through double-stranded RNA.

The binding site(s) of Tat 72 within PKR appears to be on or near the site of eIF2α binding, as preincubation of PKR with eIF2 blocks Tat binding (N. M. J. McMillan and B. R. G. Williams, unpublished observations). This suggests that Tat is acting as a substrate analogue for PKR and blocks substrate phosphorylation in this manner. This hypothesis is further supported by the fact that Tat 86 is phosphorylated by PKR *in vitro.* However, the possibility that eIF2 changes the conformation of PKR and thus obscures the Tat binding site(s) cannot be discounted. Furthermore, PKR activation, which is thought to occur via intermolecular phosphorylation, could be inhibited by interfering with PKR–PKR interaction. A known example

of a PKR inhibitor which acts as a substrate analogue is the K3L gene product of Vaccinia virus (Beattie *et al.,* 1991). K3L shares 28% identity with the α-subunit of eIF2 and lacks the essential Ser^{51} that is phosphorylated by PKR. However, Tat shares very little sequence similarity with eIF2α. Davies *et al.* (1993) have shown that K3L inhibits PKR activation as well as substrate phosphorylation without becoming phosphorylated itself. Furthermore, K3L, like Tat 72, could be coimmunoprecipitated with PKR (Carroll *et al.,* 1993).

Interestingly, Tat 86 was specifically phosphorylated by preactivated PKR, indicating that Tat 86 is a PKR substrate. However, the functional implications of PKR phosphorylation of Tat 86 remain unclear. Examination of the Tat 86 sequence indicates that there are 6 serine and 7 threonine residues that represent potential phosphorylation sites. Three of these amino acids lie within the 14 extra amino acids of the Tat 86 sequence, leading to the speculation that while both Tat 72 and Tat 86 bind to the substrate-binding region of PKR, only Tat 86 can be phosphorylated and released due to phosphorylation sites present at the C-terminus. In this manner Tat 86 would not cause direct inhibition of PKR. An alternative explanation could be that the two Tat forms have slightly different conformations, resulting in the availability of a phosphorylation site within Tat 86 which is masked in Tat 72.

Previous work has indicated that productive HIV-1 infection, or continual Tat expression, could decrease cellular PKR protein levels, but the exact mechanism by which this occurred was not determined (Roy *et al.,* 1990). In this study no reduction in the absolute levels of PKR in Tat-expressing HeLa cells was observed (Fig. 6C) and therefore appears not to be the mechanism by which Tat acts in the Tat-expressing HeLa cells described here.

PKR is activated during infection by many different viruses (including HIV) and has been hypothesized to be an important component in the establishment of the antiviral state induced by interferon (Pestka *et al.,* 1987). This has been directly demonstrated by the partial resistance of NIH3T3 cells constitutively expressing PKR to encephalomyocarditis virus infection (Meurs *et al.,* 1992). Along with other viruses which have evolved strategies to circumvent PKR activity, HIV (perhaps through the action of the Tat protein) could suppress PKR activation and thereby prevent the inhibition of protein synthesis mediated by PKR. Whether the differential effect of Tat 72 compared to Tat 86 *in vitro* has relevance in terms of the HIV-1 life cycle remains to be determined. If one-exon Tat is synthesized during the late stages of viral infection at a time when viral structural proteins accumulate, this stage of infection would represent a period in which HIV might be acutely sensitive to translation inhibition, presenting the optimal time for the virus to counteract such inhibition. By defining the Tat-binding region within PKR it may be possible to design peptide inhibitors of Tat which could prove useful in testing this hypothesis.

ACKNOWLEDGMENTS

This investigation was supported by NIAID grants, RO1 AI-34039 and AI-20611, a grant from the Human Frontier Science program to B.R.G.W and A.G.H, and the AIDS Targeted Anti-Viral program from the Office of the Director, NIH. D.P.S. was supported by a Ph.D. studentship from the MRC (Canada). We thank Drs. Robert Silverman and M. Berkirane for invaluable advice, Dr. Ian Clark-Lewis (Vancouver) for synthetic Tat protein, Drs. Michael Katze and Glen Barber for PKR polyclonal antibody, and Dr. William Merrick for mammalian eIF2. Thanks also go to Juliet Sheldon and Lorenz Truemper for technical support. Antiserum to Tat from Dr. Bryan Cullen (Hauber *et al.,* 1987) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

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